REMARKS

Applicants respectfully request reconsideration of this application in view of the above amendment and the following remarks.

Applicants have amended Claims 1, 10, 76, 85. and 139. Applicants have cancelled Claims 9, and 84.

Claims 1 and 76, have been amended to include a limitation of a growth hormone releasing hormone and protein analog thereof, and the female mammal being a farm mammal. Support for this amendment can be found in the specification paragraphs [0017], [0019] – [0023].

Pending in the application are Claims 1, 5, 6, 7, 8, 10, 12-13, 76, 81, 82, 83, 84, 87-88, and 137-139.

I. Examiner's Interview of April 20, 2006:

Applicants and the undersigned wish to thank Drs. Joanne Hama and Ram Shukla for the courtesy of granting an in-person interview at the Patent and Trademark Office on April 20, 2006. The interview was attended by Drs. Joanne Hama; Ram Shukla; Ruxandra Draghia-Akli; Craig Conrad; and the undersigned. The interview was very informative and helpful for the Applicants and the undersigned to amend the claims as given below. As suggested by Dr. Shukla, applicants have submitted a Declaration under 37 C.F.R. §1.132, prepared by one of the named inventors, Ruxandra Draghia-Akli, to address certain issues discussed during the interview.

II. Information Disclosure Statement:

Applicants acknowledge that the Examiner has considered the IDS submitted on November 14, 2005.

III. Rejections Under 35 U.S.C. §112 First Paragraph.

(A) The Examiner has maintained the rejections of Claims 1, 5-10, 12, 13, 76, 80-85, 137, and 138 under 35 U.S.C. §112 First Paragraph. The Examiner is of opinion that the specification does not reasonably provide enablement for enhancing growth in an offspring from ANY female mammal using a vector expressing any GHRH or any analog thereof during gestation. The Examiner is of the opinion that the amendment filed on November 14, 2005 was partially persuasive regarding the scope of route of vector administration by electroporation.

During the interview of April 20, 2006, the Examiner raised the issue that ghrelin may fall under the broad definition of a growth hormone related hormone or analog thereof. The Examiner also expressed concerns that certain bio-molecules, lipids, or other compositions may be construed as a growth hormone related hormone or analog thereof.

Paragraph [0052] of the specification defines:

> *[0052] The term "growth hormone releasing hormone analog" as used herein is defined as a protein which contains amino acid mutations and/or deletions in the naturally occurring form of the amino acid sequence (with no synthetic dextro or cyclic amino acids), but not naturally occurring in the GHRH molecule, yet still retains its function to enhance synthesis and secretion of growth hormone. (Emphasis Added)*

Applicants submit that one of ordinary skill in the art would have known that "ghrelin" enhances **secretion** of a growth hormone, but does NOT enhance its **synthesis**. As such, ghrelin is not a growth hormone releasing hormone analog, as defined in paragraph [0052]. See also the discussion in paragraphs [0171] and [0072] of the specification.

Applicants submit that the Anderson (Anderson et al., 2004 Exp. Biol., Med 229:291-302), which was cited by the Examiner, clearly distinguishes this difference between GHRH and ghrelin on page 292 paragraph (a) Growth Hormone Releasing Factor (GHRH); and paragraph (c) Ghrelin.

Additionally, as suggested by the Examiner, Applicants have amended independent Claims 1 and 76 to read in parts:

*"a growth hormone releasing hormone or **protein** analog thereof,"*

Thus, Applicants respectfully submit that the above amendments render moot the Examiner's rejections.

(B) The Examiner has maintained the opinion that the claims broadly encompass any female animal, while the specification teaches the effects of GHRH and HV-GHRH only on pigs and rats. Furthermore, the Examiner cited Hammer et al., 1986, which shows not all mammals "predictably" respond to expression of heterologous proteins.

During the interview of April 20, 2006, Applicants presented evidence that other species of farm mammals could be used, Thus, the Examiner suggested that all information presented at the interview be entered into the record in the form a an affidavit. The Examiner also suggested that the independent claims be limited to farm animals.

In response, Applicants have amended the claims to be limited to farm animals. Additionally, Applicants have submitted a Declaration under 37 C.F.R. §1.132 to show no undue experimentation would be involved.

Applicants submit that the Examiner's point of the Hammer et al. reference is well taken. However, it is **NOT** applicable to the current invention. Hammer et al. utilize animals with an altered genome, and Applicants invention pertains to a farm animal that is NOT genetically modified.

Applicants' invention introduces a plasmid expression vector into some of the muscle cells of a farm animal (See Amended Claim 1) using electroporation. Unlike in the classic gene therapy approaches, the vector of current invention does not need to be expressed in all cells of the mammal to elicit the claimed response. All that are needed are a few excreting cells to supply the necessary GHRH signal.

Applicants submit that the examples in the specification show that the claimed invention actually worked despite the markedly disparate placental structures of the two species (namely, pig and rat) in the examples. In pig, six membranes separate the fetal and the maternal circulations, whereas there are only three in the rat. This further confirms that this is a reproducible cross-species phenomenon contradicting Hammer's transgenic animal studies. It is interesting to note that numerous parties have used the invention described in the application and found the results to be reproducible for: (1) large animals (pigs) maintained in completely different conditions (farm conditions rather than laboratory conditions); and (2) other animal species (beef cattle and dairy cattle) maintained in farm conditions. Some of the findings were described in Brown et al., "Immune Enhancing Effects of Growth Hormone Releasing Hormone Delivered by Plasmid Injection and Electroporation," Molecular Therapy Vol. 10(4) p 644-651, (2004), a copy of which is attached as Exhibit A.

In light of the amendment and argument above, Applicants respectfully submit that Examiner's rejection is now moot.

IV. Rejections Under 35 U.S.C. §112 Second Paragraph.

Claim 139 uses redundant phrases and appears to be missing an element (i.e. a eukaryotic promoter), which is needed to drive transcription.

In response, Applicants have amended Claim 139 as suggested by the Examiner.

V. Conclusions

Applicants respectfully submit that, in light of the foregoing amendments and argument, Claims 1, 5-10, 12-13, 76, 80-85, 87-88, and 137-139 are in condition for allowance. A Notice of Allowance is therefore requested.

If the Examiner has any other matters which pertain to this Application, the Examiner is encouraged to contact the undersigned to resolve these matters by Examiner's Amendment where possible.

Respectfully submitted,

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ARTICLE

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Immune-Enhancing Effects of Growth Hormone-Releasing Hormone Delivered by Plasmid Injection and Electroporation

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Growth hormone-releasing hormone (GHRH) is a hypothalamic hormone with both direct and indirect functions in the maintenance of immune status under physiological and pathological conditions. In this study, 52 Holstein heifers were evaluated for the effects of a plasmid-mediated GHRH treatment on their immune function and on the morbidity and mortality of treated animals. In the third trimester of pregnancy, 32 heifers received 2.5 mg of a myogenic GHRH-expressing plasmid by intramuscular injection followed by electroporation, while 20 heifers were used as controls. No adverse effects were associated with either the plasmid delivery or GHRH expression. At 18 days after plasmid administration, GHRH-treated animals had increased numbers of $CD2^+$ $\alpha\beta$ T-cells ($P < 0.004$), $CD25^+CD4^+$ cells ($P < 0.0007$), and $CD4^+CD45R^+$ cells ($P < 0.016$) compared to controls. These increases were maintained long term after treatment and correlated with plasmid expression. At 300 days post-GHRH therapy, $CD45R^+/CD45R0^-$ naïve lymphocytes were significantly increased in frequency ($P < 0.05$). Natural killer lymphocytes ($CD3^-CD2^+$) were also increased. As a consequence of improved health status, body condition scores of treated animals improved (3.55 vs. 3.35, $P < 0.0001$). Hoof pathology was also reduced with treatment. The mortality of heifers was decreased (3% vs. 20% in controls, $P < 0.003$). Collectively, these results indicate that the myogenic GHRH plasmid can be successfully electrotransferred into a 500-kg mammal and expressed for prolonged periods of time, ensuring physiological levels of GHRH. The plasmid injection followed by electroporation could prove an efficient method for the systemic production of therapeutic proteins and may provide a useful means for basic research in relevant animal models.

Key Words: electroporation, GHRH, GH, IGF-I, immunity, plasmid

INTRODUCTION

Traditionally, plasmid-mediated gene transfer technology has been limited in scope because expression levels following naked DNA transfer have been low, only a fraction of that achieved after viral-mediated gene transfer [1]. Furthermore, for large mammals the doses of plasmid needed to achieve sufficient levels of circulating proteins, other than for vaccination purposes, are sometimes unrealistic as a common therapy or for basic research purposes. Conversely, results obtained in rodents are not always translated successfully to larger mammals or humans [2]. Physiologically relevant levels of plasmid DNA transfer and transgene expression can be accomplished by utilizing the electroporation technique [3–5]. Electroporation has proven useful *in vivo* in patients on whom drug delivery to malignant tumors has been performed [6]. Many recent studies of plasmid delivery enhanced with electroporation have used skeletal muscle cells as an ideal target for direct plasmid transfer [7,8] and have shown that the injected muscle can be used as a bioreactor for the persistent production and secretion of proteins at physiological levels into the bloodstream.

Our previous studies showed that delivery of a plasmid that expresses growth hormone-releasing hormone (GHRH) by intramuscular injection followed by electroporation is scalable and represents a promising approach for long-term stable production of secreted proteins in pigs and dogs [9,10].

Two hypothalamic hormones, GHRH and somatostatin, regulate the secretion of growth hormone (GH); GHRH stimulates GH release from the pituitary, and somatostatin inhibits its release, resulting in the pulsa-

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tile release of GH into the circulation. Studies in different animal models and humans have shown that GHRH has many physiological roles. Among these is a stimulatory effect on immunity, both through stimulation of the GH/insulin-like growth factor-I (IGF-I) axis and directly as an immune modulator [11,12]. Some experiments have suggested that GHRH can also modulate immune functions through brain mechanisms that are also involved in the regulation of sleep [13].

Although the cumulative observations clearly show that significant progress has been made in establishing a role for GHRH in development and regulation of the immune system, detail is still lacking on how GHRH mediates those effects. Until now, there has been no dynamic large animal model in which the biological effects of GHRH could be monitored over periods of time sufficient to measure changes in immune function and general improvement in health.

We propose that our model of delivering the hormone by plasmid injection followed by electroporation can be extended to the study of the underlying immune changes that may occur with GHRH administration. Moreover, the direct effects of GHRH plasmid administration on morbidity and mortality rates in the treated herds can be investigated. An additional advancement that has increased the potential of using large animals for GHRH studies has been the development of strong expression vectors that can be delivered at relatively low quantities (2.5 mg in animals with average weight of more than 500 kg) into muscle cells. The present study was undertaken to demonstrate the potential of the plasmid-electroporation model and answer two questions: (1) Does a plasmid-mediated GHRH therapy produce long-term beneficial effects on the immune function in cattle? (2) Are these changes clinically relevant? The results of our study demonstrate that plasmid-mediated GHRH treatment stimulates an increase in T-cell and natural killer cells in peripheral blood that appears to be associated clinically with an improvement in immune function. The clinical consequences are reduced morbidity and mortality and improved body condition scores in the treated heifers compared to controls.

RESULTS

Biochemistry and CBC Values

At 300 days posttreatment, the total white blood cell counts were similar between groups. Nevertheless, the percentage of circulating lymphocytes was increased in GHRH-treated animals (47.4 ± 3.3% vs. controls, 37.8 ± 5.3%, $P < 0.06$). We also observed a physiological increase in hemoglobin (11.55 ± 0.15 g/dl in GHRH-treated vs. 10.9 ± 0.15 g/dl in controls, $P < 0.02$) and red blood cells (7.65 ± 0.1 million/ml vs. 7.3 ± 0.2 million/ml, $P < 0.07$) at this time point. We found no differences between the groups in other CBC or serum biochemistry panels at any time point tested. These were within the normal range of values for cattle. Glucose and insulin levels were not different between groups (Table 1).

TABLE 1: Glucose and insulin levels in GHRH-treated animals and controls

	GHRH-treated	Control	*P* value
Glucose, mg/dl			
60 DIM	43.9 ± 1.4	41.8 ± 4.5	0.67
100 DIM	72.2 ± 1.4	69.3 ± 3.8	0.5
Insulin, μU/ml			
60 DIM	11.6 ± 2.5	9.4 ± 3.2	0.59
100 DIM	7.8 ± 1.5	7.4 ± 0.6	0.8

Glucose and insulin levels were measured at 60 and 100 DIM. Values are presented as means ± SEM.

Immune Markers

The total number of white blood cells, differentials, and flow cytometric (FC) profiles were similar between groups at day 0. At 18 days posttreatment, $CD2^+$ values were increased in treated animals by 14%, but there was no change in controls compared to baseline values: 43.4 ± 1.7% vs. 37.9 ± 1.4%, $P < 0.004$, in GHRH-treated cattle and 37.3 ± 2.1% vs. 38.8 ± 1.8% in controls (Fig. 1A). The $CD4^+/CD8^+$ ratio increased in treated animals (day 18–day 0 = 8 ± 0.6%, $P < 0.04$), mostly due to an increase in $CD4^+$ cells (29.1 ± 0.7% at day 18 vs. 24.5 ± 0.8% at day 0). During the same period $CD4^+CD45R^+$ naïve lymphocytes increased by 53% with the GHRH treatment: day 18, 11.1 ± 0.4% vs. day 0, 7.4 ± 0.4% in GHRH-treated animals, $P < 0.016$, and day 18, 8 ± 0.7% vs. day 0, 7.2 ± 0.8% in control animals (Fig. 1B). $CD25^+CD4^+$ cells were also significantly increased with treatment: day 18, 4.3 ± 0.3% vs. day 0, 1 ± 0.1 in GHRH-treated animals, $P < 0.001$, and day 18, 3.8 ± 0.2% vs. day 0, 1.7 ± 0.2 in controls.

At 300 days posttreatment, when a more comprehensive panel was performed, we found that $CD45R^+/CD45R0^-$ naïve lymphocytes were significantly more numerous in treated animals (0.98 ± 0.08) than in controls (0.91 ± 0.08), $P < 0.05$ (Fig. 1C). $CD2^+CD3^+\gamma\delta^-$ cells were more numerous with treatment: 68.5 ± 1.4% of all $CD2^+$ cells vs. 60 ± 5.6% in controls, $P < 0.02$.

Mortality in Treated Animals

The mortality of the heifers (involuntary cull rate) was different between GHRH-treated animals and controls. During the 360-day study, none of the treated heifers died, while 20% of control heifers had to be culled ($P < 0.003$). The causes of death were the following: one Johne's disease, one systemic infection from hoof conditions and an infected cut, one animal with severe hoof problems complicated by rear leg paralysis, and one severe mastitis case. One treated animal was culled

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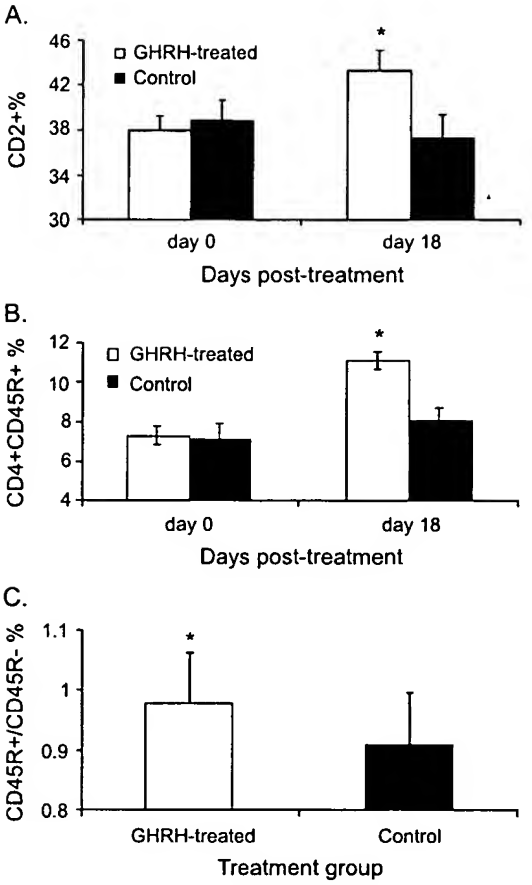

FIG. 1. Percentage of (A) $CD2^+$ cells and (B) $CD4^+CD45R^+$ naïve T-cells at days 0 and 18 after treatment and of (C) the ratio of $CD45R^+/CD45R^-$ naïve T-cells at 300 days posttreatment. Values are presented as means ± SEM, $^*P < 0.001$.

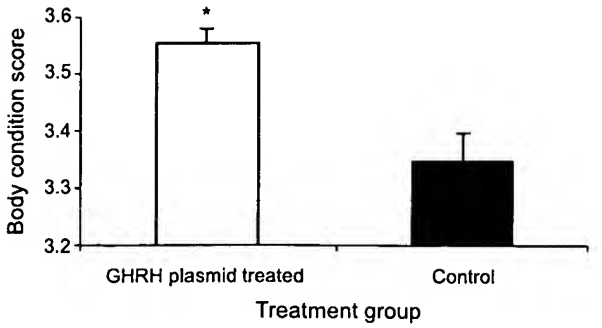

FIG. 2. Body condition scores in heifers treated with pSP-HV-GHRH versus controls at 60 to 80 DIM. Body condition scores differed between treatment groups, $P < 0.0001$.

due to an accident. The overall involuntary cull rate prior to 120 days in milk production (DIM) was decreased by 40% with the treatment.

Body Weights and Body Condition Score

Body condition scores (BCS) of heifers differed between groups at the time of stress and negative energy balance, at 60 to 80 DIM. Heifers treated with pSP-HV-GHRH showed an improvement ($P < 0.0001$, Fig. 2) in BCS between 60 and 80 DIM. During the first 100 DIM, treated animals lost an average of 3.5 kg (0.06% of total body weight) ($P < 0.02$, Table 2) while control cows lost on average of 26.4 kg (4.6% of body weight at 60 DIM). The better BCS correlated with an increase in the serum IGF-I levels: day 100–day 60 = 22.4 ± 4 ng/ml for GHRH-treated heifers (119.7 ± 6.9 ng/ml at day 100 vs. 97.3 ± 6.6 ng/ml at day 60) vs. 8 ± 7.4 ng/ml for controls (99.8 ± 3.9 ng/ml at day 100 vs. 91.8 ± 6.8 ng/ml at day 60) ($P < 0.04$).

Morbidity

The herd had significant hoof pathology at the beginning of the study prior to plasmid administration. Foot problems, most probably of bacterial origin [14], were also one of the principal causes of morbidity in these animals throughout the study. The proportion of animals that had worsening foot problems throughout the course of the study was 40% higher for controls compared to the treated animals: 7 of 32 GHRH-treated animals versus 7 of 20 controls. The overall hoof score improvement did not reach statistical significance ($P < 0.4$) due to high interanimal variability in the control group.

DISCUSSION

Recently, significant progress has been made to enhance plasmid delivery *in vivo* and subsequently to achieve physiological levels of a secreted protein. Intramuscular injection of plasmid followed by electroporation has been used successfully in ruminants for

TABLE 2: Weight of heifers 60 days precalving (DPC) and 100 and 135 DIM, weight difference between these time points, and percentage difference compared to baseline weight

	GHRH-treated	Control	*P* value
Weight (kg)			
60 DPC (A)	540 ± 28.1	543 ± 42.7	0.38
100 DIM (B)	536.4 ± 32.9	514.2 ± 48	**0.04**
135 DIM (C)	544.9 ± 33.1	533.5 ± 44.8	0.17
Difference (kg)			
A-B	−3.5	−26.4	**0.02**
A-C	4	−9.1	0.1
Difference (%)			
A-B/A	−0.5%	−4.6%	**0.02**
A-C/A	0.1%	−1.4%	0.1

Values are presented as means ± SEM. *P* value for each specific comparison between treatments is given.

vaccination purposes [15,16]. We have demonstrated previously in mice, rats, pigs, and dogs that GHRH can be efficiently produced by muscle cells and released into the circulation using this technology. Here, we apply this method to a cattle model to study the immune-enhancing effects of GHRH, and we show that the plasmid injection/electroporation is scalable and provides the framework for potential human applications of this technology [17]. Furthermore, we have followed expression for more than 300 days after a single administration of GHRH plasmid, demonstrating the long-term applicability of this treatment system.

Stimulation of the GH axis has been shown to have a variety of positive immunostimulatory effects [18]. A substantial body of research exists that supports the production of GHRH, GH, and IGF-I by cells of the immune system [19], suggesting that immune function might be regulated by both autocrine and paracrine mechanisms. It has also been suggested that the increased morbidity in the elderly may be causally related to two changes that occur with aging, decreased GH/IGF-I production and decreased immune function [20].

Studies have shown that administration of GHRH or its analogs in the elderly resulted in profound immunoenhancing effects, both short- and long-term after therapy, with increased numbers of lymphocytes, monocytes, B-cells and cells expressing T-cell receptor $\alpha\beta$, and T-cell receptor $\gamma\delta$ [21].

GH secretagogues have been shown to inhibit the initiation of tumors in aged mice [22] in parallel with increased thymic cellularity. Debilitated dogs with cancer that received plasmid-mediated GHRH had improved immune parameters and red blood cell production [23]; the improved quality of life of the dogs was maintained for at least 1 year after a single plasmid administration [24]. Furthermore, overexpression of heterologous or homologous GH in transgenic mice led to significant stimulation of some parameters of immune function, such as increases in the absolute weight of the thymus and the spleen and in the mitogenic responses of splenocytes to concanavalin A, lipopolysaccharide, and phytohemagglutinin [12].

IGF-I modulates immune function and has two major effects on B-cell development, with potentiation and maturation, and acts as a cofactor for B-cell proliferation. The recombinant protein increases the number of pre-B- and mature B-cells in bone marrow [25]. The mature B-cell remains sensitive to IGF-I [26].

It has been generally assumed that plasmids that contain CpG motifs are potent agents for inducing inflammatory cytokines *in vivo* that, in turn, promote activation of transgene product-specific B- and T-cells [27]. The plasmid pSP-HV-GHRH contains a total of 183 CpG sequences. Of these, 93 are part of a class shown to be nonimmunostimulatory or even to suppress the effects of immunostimulatory CpGs (so-called CpG-N motifs, including CCG, CGG, and CGCG sequences as reviewed in [28]). The remaining 90 CpGs are consistent with immune-stimulatory sequences (CpG-S motifs). The specific effect, if any, of these CpG motifs on the biological activity of plasmid is unknown and will require further investigation. While control animals did not receive empty plasmid, it is unlikely that the described immune stimulation may have been nonspecific and mediated through the CpG motifs present in the plasmid backbone, considering the long-term hormonal and body composition changes in the GHRH-treated animals.

This study demonstrated a correlation between the improved health status of GHRH-treated heifers and changes in the composition of peripheral blood mononuclear cells (PBMC) over the time investigated. Differences were noted in the frequency of $CD2^+$ T-cells, the $CD4^+/CD8^+$ ratio, and the frequency of $CD45R^+$ naïve T-cells. The clinical consequences of enhanced immune function were reduced morbidity and mortality in GHRH-treated cows. Further studies are needed to determine if improvement in immune function is the result of direct interaction of GH or IGF-I with T-cells (mediated through GHRH) or the result of indirect effects resulting from an interaction with monocytes and antigen-presenting cells [29]. GH appears to affect cell function by promoting the survival of progenitor cells in the thymus and also T-cell function in peripheral lymphoid tissue. GH and IGF-I have been reported to increase T-cell function *in vitro*. Data are limited, especially in large animals such as cattle. Our finding of an increase in $CD2^+$ T-cells, possibly associated with the increase in naïve T-cells, is consistent with an effect on the thymus and an increase in output of naïve $CD4^+$ ($CD45R^+$) T-cells. It is known that T-cell activation depends in part on the number of T cell receptors (TCR), which is further dependent on small co-stimulatory molecules, such as CD2. CD2 can significantly reduce the threshold of triggered TCRs required for cell proliferation and cytokine synthesis. As a consequence, CD2 stimulates T-cell activation in the absence of direct engagement of antigen-specific TCR and increases expression of the TCR ζ chain, resulting in enhanced CD3 receptor density [30], and cytokine production [31]. Enhanced TCR and CD3 constitutive expression was reported to be a factor in innate immunity or autoimmunity [32]. Further studies are now needed to determine if the subtle changes shown in our study are associated with changes in the capacity of naïve T-cells to respond to pathogenic organisms. The potential that needs to be explored is whether these types of effects could also influence maturation of the immune system in the fetus and result in enhanced survival as noted in these studies.

Methods that could decrease the rate of animal loss under farm conditions, improve BCS, and reduce pathology in the herd are highly desirable. A current approach used to increase herd productivity is admin-

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istration of bovine somatotrophin (bST). However, bST administration to cows does not affect the animal's body weight, BCS, and energy balance at conception and during pregnancy [33,34] and may have detrimental effects in some cases. Some undesirable side effects of GH protein therapies may be due to the fact that treatment with recombinant exogenous GH protein delivers only one GH isoform, abolishing the natural episodic pulses of GH, and inducing supraphysiological levels of GH. By contrast, no side effects have been reported for recombinant GHRH therapies. We confirmed this finding long term with glucose and insulin levels, which can be abnormal during GH therapies, but were normal throughout this study.

Although recombinant GHRH protein therapy stimulates normal cyclical GH secretion with virtually no side effects, the short half-life of GHRH *in vivo* requires frequent administration [35,36], and the therapy is expensive and clinically impractical. A plasmid-mediated method could overcome this limitation to GHRH use. Because of the physiological feedback regulation of the GHRH–GH–IGF-I axis in normal mammals, a wide range of plasmid doses may be efficacious.

In the present study, GHRH-treated dairy cattle had decreased morbidity and mortality rates. Culling (mortality) is a major economic problem in the farm animal industry. There are many reasons for culling animals, separated into involuntary (persistent hoof problems, persistent mastitis, nonbreeders, disease, or death) and voluntary culling (breeding stock or lower producing animals). Due to the high percentage of involuntary culling, voluntary cull decisions revolving around rational economic parameters (e.g., maintenance of herd size) are typically held to a minimum. The average overall cull rate in North America is approximately 36% [37]. In our case, the cull rate was 20% for controls, while only 1 of the 32 GHRH-treated animals was culled, due to an accident.

The digital dermatitis lesions that constituted the major hoof pathology were attenuated by the GHRH treatment. Studies have shown that as much as 29% of dairy cattle and 4% of beef cattle have gross lesions of digital dermatitis and that spirochetes are involved in more than 60% of the cases [38]. The immune response to the spirochetes is of short duration [39], thus, to diminish the infection burden, a stable long-term therapy would be preferable.

Previous work showed that changes in serum IGF-I postpartum may help predict both nutritional and reproductive status in dairy cattle [40]. Also, it has been shown that concentrations of IGF-I in serum are associated with BCS [41]. This association was confirmed in our study, with IGF-I levels that were maintained in treated animals throughout stress periods and correlated to increased immune surveillance and body mass.

Body condition scores and maintenance of body weight are indicators of adequate nutrition, breeding ability, and recognition of health status in dairy herds [42–44]. Body condition is a reflection of the body fat reserves carried by the animal [45,46]. These reserves can be used by the cow during early lactation, when the animals tend to be in a negative energy balance, with a loss of body condition and decrease in body weight [42,47]. In early lactation, body condition scores between 3.4 and 3.7 are considered in the optimum range. Minimized BCS loss translates to decreased mobilization of body tissue, resulting in better health status and productivity. As shown in the present experiment, between 60 and 80 DIM, control animals showed a significant decrease in total body weight and BCS, while treated cows maintained their initial weight and BCS, which may explain their reduced morbidity and mortality.

In this study, decreased morbidity and culling rates, increased BCS, and positive changes in the immune system have been achieved in a large animal with a single plasmid-mediated GHRH treatment. These clinical effects

TABLE 3: Monoclonal antibodies used to specify peripheral blood mononuclear cells

mAb	Ig isotype	Specificity
MUC2A [52]	IgG2a	CD2 expressed on αβ T-cells, a subset of γδ T-cells, and a $CD2^+CD3^-$ population of lymphocytes containing NK cells
BAT76 [52]	IgG2a	CD2 as noted above
MM1A [53]	IgG1	CD3 T-cell receptor expressed on αβ and γδ T-cells
ILA-11A [54]	IgG2a	CD4 expressed on T-helper/inducer T-cells
CACT138A [52]	IgG1	CD4 as noted above
CACT187A[a]	IgG1	CD4 as noted above
B29A [55]	IgG2a	CD5 expressed on αβ and γδ T-cells and a subset of B-cells
7C2B[a]	IgG2a	CD8α expressed on cytotoxic/immunomodulatory T-cells
CACT80C [52]	IgG1	CD8α as noted above
BAT82A [52]	IgG1	CD8β as noted above
CACT116A [56]	IgG1	CD25 interleukin-2Rα expressed on activated T-cells
CACT114A [57]	IgG2b	CD26 dipeptidylpeptidase IV expressed on activated memory T-cells
GS5A [58]	IgG1	CD45R expressed on B-cells, naïve T-cells, and a subset of $CD45R0^+$ memory T-cells
GC44A1 [58]	IgG3	CD45R0 expressed on granulocytes, monocytes, memory T-cells, and γδ T-cells
GB21A [59]	IgG2b	γδ T-cells, δ-chain specific
BAQ44A [60]	IgM	Expressed on B-cells (CD specificity not determined)
DH59B [61]	IgG1	CD172a expressed on monocytes and granulocytes

[a] Unpublished.

were maintained both short-term and long-term after treatment.

Materials and Methods

DNA constructs. The plasmid pSPc5-12 contains a 360-bp *SacI*/*Bam*HI fragment of the SPc5-12 synthetic promoter [48] in the *SacI*/*Bam*HI sites of a pSK-GHRH backbone. The synthetic GHRH cDNA, HV-GHRH, was obtained by site-directed mutagenesis of porcine GHRH cDNA (1–40)OH substitutions of Tyr to His at position 1, Ala to Val at position 2, Gly to Ala at position 15, Met to Leu at position 27, and Ser to Asn at position 28 using the Altered Sites II *in Vitro* Mutagenesis System (Promega, Madison, WI, USA) and cloned into the *Bam*HI/*Hin*dIII sites of pSK-GHRH. The GHRH cDNA is followed by the 3′ untranslated region of GH.

Animals. Thirty-two primiparous Holstein cows, 18 to 20 months of age, with an average weight of 547 ± 43 kg, were treated with 2.5 mg pSP-HV-GHRH once during the last trimester of gestation and designated as the treated group. Similarly, 20 pregnant heifers from the same source and of the same breed and age did not receive plasmid treatment and served as controls. Animals calved at age 23 months ± 24 days. Cows were housed in a free-stall barn fitted with fans equipped with water misters for evaporative cooling and exposed to natural daylight. The herd was fed a silage-based total mixed ration *ad libitum* twice daily. Each cow was fitted with a transponder/pedometer that allowed for automatic identification upon entering the stall. At the conclusion of this experiment, all animals treated with plasmid were disposed of in such a manner that their tissues did not enter the food chain. All milk and tissues produced by treated animals were destroyed and did not enter the human food chain. Animal protocols were conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

Intramuscular injection of plasmid DNA. The endotoxin-free plasmid (Qiagen, Inc., Chatsworth, CA, USA) preparation of pSPc5-12-HV-GHRH was diluted in water to 5 mg/ml and formulated with poly-L-glutamate 1% wt/wt. Cows were given a total quantity of 2.5 mg pSP-HV-GHRH intramuscularly in the trapezius muscle using a 21-gauge needle (Becton–Dickinson, Franklin Lakes, NJ, USA). Two minutes after injection, the injected muscle was electroporated, 5 pulses, 1 A, 52 ms/pulse, as described [49]. The voltage changes with the change in resistance of the tissue during

TABLE 4: Combinations of mAbs used in three-color FC to determine the composition and frequency of different populations of peripheral blood mononuclear cells and the functional status of CD4 and CD8 αβ T-cells and γδ T-cells

mAb combination	Specificity	Gating strategy used to obtain data on cell subsets
MM1A	CD3	(1) Analyzed ungated to determine the frequency of the $CD3^-CD2^+$ cell population containing NK cells. (2) Analyzed with a gate on $CD3^+$ T-cells to determine the frequency of $CD2^+$ αβ T-cells, $CD2^+$ γδ T-cells, and $CD2^-$ γδ T-cells present in blood.
MUC2A	CD2	
BAT76A	CD2	
GB21A	γδ	
ILA-11A	CD4	Analyzed with a gate on CD4 to determine the frequency of $CD4^+CD45R^+$ naïve and $CD4^+CD45R^+$ memory and $CD45R0^+$ memory T-cells present in peripheral blood.
GS5A	CD45R	
GC44A1	CD45R0	
ILA-11A	CD4	Analyzed with a gate on CD4 to determine the frequency of $CD4^+$ naïve and memory T-cells expressing the activation molecule CD25.
CACT116A	CD25	
GC44A1	CD45R0	
CACT138A	CD4	Analyzed with a gate on CD4 to determine the frequency of $CD4^+$ naïve and memory T-cells expressing the activation molecule CD26.
CACT187A	CD4	
CACT114A	CD26	
GC44A1	CD45R0	
7C2B	CD8	Analyzed with a gate on CD8 to determine the frequency of $CD4^+CD45R^+$ naive and $CD4^+CD45R^+$ memory and $CD45R0^+$ memory T-cells present in peripheral blood.
GS5A	CD45R	
GC44A1	CD45R0	
CACT116A	CD25	Analyzed with a gate on CD8 to determine the frequency of $CD8^+$ naïve and memory T-cells expressing the activation molecule CD25.
GC44A1	CD45R0	
CACT80CA	CD8	Analyzed with a gate on CD8 to determine the frequency of $CD8^+$ naïve and memory T-cells expressing the activation molecule CD26.
BAT82AA	CD8	
CACT114A	CD26	
GC44A1	CD45R0	
GB21A	γδ	Analyzed with a gate on γδ T-cells to determine the frequency of γδ T-cells expressing CD25.
CACT116A	CD25	
GC44A1	CD45R0	
B29A	CD5	Analyzed with no gates to determine the frequency of αβ and γδ $CD5^+$ T-cells, $CD5^+$ and $CD5^-$ B-cells, and monocytes.
BAQ44A	B	
DH59B	CD172a	

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the electroporation (to maintain constant current), and it has been recorded to be between 80 and 120 V/cm. For all injections, 2-cm needles were inserted through the skin into the muscle. Animals were observed immediately after injection and 24 h later for any adverse effects at the electroporation site.

Weight, body condition, and hoof scores. Before treatment, heifers were weighed on the same calibrated scale (Priefert cattle squeeze-chute connected to a Weigh Tronix 915A indicator and WP233 printer; Central City Scale, Central City, NE, USA) and randomly assigned to groups. Two independent dairy animal scientists (Texas A&M University), blinded to the treatment groups, assessed body condition scores prior to treatment, between 60 and 80 DIM, and between 100 and 120 DIM. Cows were scored by both observing and handling the backbone, loin, and rump areas [50], with possible BCS ranging from 1 (very thin cow) to 5 (a severely overconditioned cow). Hoof scores were measured prior to plasmid-GHRH treatment and at 60 DIM. Hoof scores included a 0 (no hoof problem) to 4 (severe hoof problem) evaluation of each foot. Each hoof was assigned an additional 2 points for abscesses eventually present (1 point) and the necessary treatments at any given time (1 point). Possible hoof scores ranged from 0 (no problem) to 24 (severe hoof problems at all 4 feet, abscesses, and intense treatment needed for each hoof).

Complete blood counts and immune markers. Whole blood from all heifers was collected in EDTA and submitted for complete blood count analysis (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX, USA) prior to treatment and at 18 and 300 days posttreatment. Hematology parameters included erythrocyte counts, hematocrit, hemoglobin, total leukocyte count, and differential leukocyte counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and fibrinogen.

Immune markers were assayed on all treated cattle and controls at days 0 and 18 and on 20 treated and 10 control cows at 300 days posttreatment. Analysis was performed by FC using monoclonal antibodies (mAb) developed in Dr. Davis's laboratory (Department of Veterinary Microbiology/Pathology, CVM, Washington State University, Pullman, WA, USA). Table 3 shows the immunoglobulin isotype and specificity of the mAbs used in the study. Table 4 shows the combinations of mAbs used in three-color FC to determine the composition and frequency of different populations of PBMC in peripheral blood and the functional status of CD4 and CD8 $\alpha\beta$ T-cells and $\gamma\delta$ T-cells. As shown, two mAbs of the same specificity have been used in some combinations to increase the intensity of the fluorescent signal on CD4 and CD8 T cells. Ten milliliters of blood was obtained at the times indicated and processed for FC [51]. The blood was lysed in Tris-buffered NH_4Cl, washed in phosphate-buffered saline containing acid citrate dextrose (PBS/ACD), and then distributed in 96-well conical bottom tissue culture plates containing the different combinations of mAbs. The cells were incubated for 15 min on ice, washed 3× in PBS/ACD, and incubated another 15 min with different combinations of isotype-specific goat anti-mouse antibodies conjugated with fluorescein, phycoerythrin, or phycoerythrin-Cy5 (Southern Biotechnology Associates, Birmingham, AL, USA; Caltag Laboratories, Burlingame, CA, USA). The cells were then washed 2× and fixed in PBS-buffered 2% formaldehyde. The cells were kept in the refrigerator until examined on a Becton–Dickinson FACSort equipped with CellQuest software. Data were analyzed on Flow Jo (Tree Star, Inc., San Carlos, CA, USA) and FCS Express (De Novo Software, Thornton, ON, Canada) software. Unless otherwise stated, data are presented as percentages of the total population assayed with a particular monoclonal antibody or combination thereof (e.g., total $CD4^+$ and $CD4^-$ cells represent 100% of cells assayed; total $CD2^-CD3^-$, $CD2^-CD3^+$, $CD2^+CD3^-$, or $CD2^+CD3^+$ represent 100% of cells assayed with both CD2 and CD3 antibody, etc.).

Biochemistry and insulin measurements. Serum samples were collected at 60 and 100 DIM. Serum was aliquoted for radioimmunoassay and biochemical analysis and stored at −80°C prior to analysis. Biochemical analysis occurred within 48 h after serum collection (Texas Veterinary Medical Diagnostic Laboratory). Serum biochemical endpoints included alanine aminotransferase, γ-glutamyltransferase, creatine phosphokinase, total bilirubin, total protein, albumin, globulin, blood urea nitrogen, creatinine, phosphorus, calcium, and glucose. Insulin and IGF-I assays were performed within 90 days after serum collection. Samples were analyzed for glucose and insulin levels by an independent laboratory (Texas Veterinary Medical Diagnostic Laboratory). All samples were analyzed in the same assay. The assay variability was 3.6% for the insulin assay and 4.4% for the glucose assay. Total proteins were measured using a Bio-Rad protein assay kit on the serum samples (Bio-Rad Laboratories, Hercules, CA, USA).

Radioimmunoassay for IGF-I. Serum IGF-I was measured using a heterologous human immunoradiometric assay kit following the manufacturer's protocol (Diagnostic System Labs, Webster, TX, USA). The kit employs an extraction step to remove binding protein interference. All samples were run in the same assay. The intra-assay variability was 4%. Cross-reactivity of human IGF-I antibody for bovine IGF-I is 100%.

Statistical analysis. Data consisted of repeated measures at different time points with unequal allocation of experimental units to treatment groups (treated $n = 32$, controls $n = 20$). Additional comparisons were performed when a significant ($P < 0.05$) treatment × day interaction was detected. A mixed model using SAS (analysis of simple main effects) was used to examine if there were any significant differences among the groups of each variable at different time points. Categorical data, such as culling rate and hoof problems, were analyzed by ANOVA. Data were coded with numerical values such that ANOVA could be performed. The total hoof score for each animal was used in the analysis of this parameter. For mortality rates, we developed an equivalent scoring system: alive = 1, dead = 0. Serum IGF-I was analyzed by ANOVA for repeated measures. Values compared with Student's t test, ANOVA, or linear regression are presented under Results, with $P < 0.05$ taken as the level of statistical significance.

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REFERENCES

1. Manthorpe, M., *et al.* (1993). Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* 4: 419–431.
2. Fewell, J. G., *et al.* (2001). Gene therapy for the treatment of hemophilia B using PINC-formulated plasmid delivered to muscle with electroporation. *Mol. Ther.* 3: 574–583.
3. Aihara, H., and Miyazaki, J. (1998). Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol.* 16: 867–870.
4. Mathiesen, I. (1999). Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther.* 6: 508–514.
5. Mir, L. M., Bureau, M. F., Rangara, R., Schwartz, B., and Scherman, D. (1998). Long-term, high level in vivo gene expression after electric pulse-mediated gene transfer into skeletal muscle. *C.R. Acad. Sci. III* 321: 893–899.
6. Heller, R., Gilbert, R., and Jaroszeski, M. J. (1997). Electrochemotherapy: an emerging drug delivery method for the treatment of cancer. *Adv. Drug Delivery Rev.* 26: 185–197.
7. Stoll, S. M., and Calos, M. P. (2002). Extrachromosomal plasmid vectors for gene therapy. *Curr. Opin. Mol. Ther.* 4: 299–305.
8. Mor, G., and Eliza, M. (2001). Plasmid DNA vaccines: immunology, tolerance, and autoimmunity. *Mol. Biotechnol.* 19: 245–250.
9. Draghia-Akli, R., Ellis, K. M., Hill, L. A., Malone, P. B., and Fiorotto, M. L. (2003). High-

efficiency growth hormone releasing hormone plasmid vector administration into skeletal muscle mediated by electroporation in pigs. *FASEB J.* 17: 526–528.
10. Draghia-Akli, R., Cummings, K. K., Khan, A. S., Brown, P. A., and Carpenter, R. H. (2003). Effects of plasmid-mediated growth hormone releasing hormone supplementation in young healthy beagle dogs. *J. Anim. Sci.* 81: 2301–2310.
11. Khorram, O., Garthwaite, M., and Golos, T. (2001). The influence of aging and sex hormones on expression of growth hormone-releasing hormone in the human immune system. *J. Clin. Endocrinol. Metab.* 86: 3157–3161.
12. Dialynas, E., Brown-Borg, H., and Bartke, A. (1999). Immune function in transgenic mice overexpressing growth hormone (GH) releasing hormone, GH or GH antagonist. *Proc. Soc. Exp. Biol. Med.* 221: 178–183.
13. Marshall, L., Perras, B., Fehm, H. L., and Born, J. (2001). Changes in immune cell counts and interleukin (IL)-1beta production in humans after a somnogenically active growth hormone-releasing hormone (GHRH) administration. *Brain Behav. Immun.* 15: 227–234.
14. Murray, R. D., *et al.* (1996). Epidemiology of lameness in dairy cattle: description and analysis of foot lesions. *Vet. Rec.* 138: 586–591.
15. Tollefsen, S., *et al.* (2003). DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. *Scand. J. Immunol.* 57: 229–238.
16. Babiuk, L. A., Pontarollo, R., Babiuk, S., Loehr, B., and Littel-van den Hurk, v. D. (2003). Induction of immune responses by DNA vaccines in large animals. *Vaccine* 21: 649–658.
17. Khan, A. S., *et al.* (2003). Optimization of electroporation parameters for the intramuscular delivery of plasmids in pigs. *DNA Cell Biol.* 22: 807–814.
18. Dorshkind, K., and Horseman, N. D. (2001). Anterior pituitary hormones, stress, and immune system homeostasis. *Bioessays* 23: 288–294.
19. Burgess, W., *et al.* (1999). The immune–endocrine loop during aging: role of growth hormone and insulin-like growth factor-I. *Neuroimmunomodulation* 6: 56–68.
20. Gelato, M. C. (1996). Aging and immune function: a possible role for growth hormone. *Horm. Res.* 45: 46–49.
21. Khorram, O., Yeung, M., Vu, L., and Yen, S. S. (1997). Effects of [norleucine27]-growth hormone-releasing hormone (GHRH) (1–29)-NH_2 administration on the immune system of aging men and women. *J. Clin. Endocrinol. Metab.* 82: 3590–3596.
22. Koo, G. C., *et al.* (2001). Immune enhancing effect of a growth hormone secretagogue. *J. Immunol.* 166: 4195–4201.
23. Draghia-Akli, R., Hahn, K. A., King, G. K., Cummings, K., and Carpenter, R. H. (2002). Effects of plasmid mediated growth hormone releasing hormone in severely debilitated dogs with cancer. *Mol. Ther.* 6: 830–836.
24. Tone, C. M., Cardoza, D. M., Carpenter, R. H., and Draghia-Akli, R. (2004). Long-term effects of plasmid-mediated growth hormone releasing hormone in dogs. *Cancer Gene Ther.* 11: 389–396.
25. Jardieu, P., Clark, R., Mortensen, D., and Dorshkind, K. (1994). In vivo administration of insulin-like growth factor-I stimulates primary B lymphopoiesis and enhances lymphocyte recovery after bone marrow transplantation. *J. Immunol.* 152: 4320–4327.
26. Robbins, K., McCabe, S., Scheiner, T., Strasser, J., Clark, R., and Jardieu, P. (1994). Immunological effects of insulin-like growth factor-I—enhancement of immunoglobulin synthesis. *Clin. Exp. Immunol.* 95: 337–342.
27. Reyes-Sandoval, A., and Ertl, H. C. (2004). CpG methylation of a plasmid vector results in extended transgene product expression by circumventing induction of immune responses. *Mol. Ther.* 9: 249–261.
28. Krieg, A. M. (1999). Mechanisms and applications of immune stimulatory CpG oligodeoxynucleotides. *Biochim. Biophys. Acta* 1489: 107–116.
29. Welniak, L. A., Sun, R., and Murphy, W. J. (2002). The role of growth hormone in T-cell development and reconstitution. *J. Leukocyte Biol.* 71: 381–387.
30. Lin, H., Martelli, M. P., and Bierer, B. E. (2001). The involvement of the proto-oncogene p120 c-Cbl and ZAP-70 in CD2-mediated T cell activation. *Int. Immunol.* 13: 13–22.
31. LeGuiner, S., *et al.* (1998). LFA-3 co-stimulates cytokine secretion by cytotoxic T lymphocytes by providing a TCR-independent activation signal. *Eur. J. Immunol.* 28: 1322–1331.
32. Nicolas, L., *et al.* (2001). Human gammadelta T cells express a higher TCR/CD3 complex density than alphabeta T cells. *Clin. Immunol.* 98: 358–363.
33. Gallo, G. F., and Block, E. (1990). Effects of recombinant bovine somatotropin on nutritional status of dairy cows during pregnancy and of their calves. *J. Dairy Sci.* 73: 3266–3275.
34. Radcliff, R. P., *et al.* (2000). Effects of diet and injection of bovine somatotropin on prepubertal growth and first-lactation milk yields of Holstein cows. *J. Dairy Sci.* 83: 23–29.
35. Evans, W. S., *et al.* (1985). Effects of intravenous, subcutaneous, and intranasal administration of growth hormone (GH)-releasing hormone-40 on serum GH concentrations in normal men. *J. Clin. Endocrinol. Metab.* 61: 846–850.
36. Thorner, M. O., *et al.* (1986). Clinical studies with GHRH in man. *Horm. Res.* 24: 91–98.
37. Radke, B., and Shook, G. (2001). *Culling and Genetic Improvement Programs for Dairy Herds,* 3rd ed., Vol. 8, Saunders, Philadelphia, pp. 291–307 .
38. Brown, C. C., Kilgo, P. D., and Jacobsen, K. L. (2000). Prevalence of papillomatous digital dermatitis among culled adult cattle in the southeastern United States. *Am. J. Vet. Res.* 61: 928–930.
39. Trott, D. J., *et al.* (2003). Characterization of Treponema phagedenis-like spirochetes isolated from papillomatous digital dermatitis lesions in dairy cattle. *J. Clin. Microbiol.* 41: 2522–2529.
40. Zulu, V. C., Sawamukai, Y., Nakada, K., Kida, K., and Moriyoshi, M. (2002). Relationship among insulin-like growth factor-I, blood metabolites and postpartum ovarian function in dairy cows. *J. Vet. Med. Sci.* 64: 879–885.
41. Spicer, L. J., Chase, C. C., Jr., and Rutter, L. M. (2002). Relationship between serum insulin-like growth factor-I and genotype during the postpartum interval in beef cows. *J. Anim. Sci.* 80: 716–722.
42. Dechow, C. D., Rogers, G. W., and Clay, J. S. (2002). Heritability and correlations among body condition score loss, body condition score, production and reproductive performance. *J. Dairy Sci.* 85: 3062–3070.
43. Domecq, J. J., Skidmore, A. L., Lloyd, J. W., and Kaneene, J. B. (1997). Relationship between body condition scores and milk yield in a large dairy herd of high yielding Holstein cows. *J. Dairy Sci.* 80: 101–112.
44. Studer, E. (1998). A veterinary perspective of on-farm evaluation of nutrition and reproduction. *J. Dairy Sci.* 81: 872–876.
45. Apple, J. K., Davis, J. C., Stephenson, J., Hankins, J. E., Davis, J. R., and Beaty, S. L. (1999). Influence of body condition score on carcass characteristics and subprimal yield from cull beef cows. *J. Anim. Sci.* 77: 2660–2669.
46. Komaragiri, M. V., Casper, D. P., and Erdman, R. A. (1998). Factors affecting body tissue mobilization in early lactation dairy cows. 2. Effect of dietary fat on mobilization of body fat and protein. *J. Dairy Sci.* 81: 169–175.
47. De Rouen, S. M., *et al.* (1994). Prepartum body condition and weight influences on reproductive performance of first-calf beef cows. *J. Anim. Sci.* 72: 1119–1125.
48. Li, X., Eastman, E. M., Schwartz, R. J., and Draghia-Akli, R. (1999). Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nat. Biotechnol.* 17: 241–245.
49. Draghia-Akli, R., Khan, A. S., Cummings, K. K., Parghi, D., Carpenter, R. H., and Brown, P. A. (2002). Electrical enhancement of formulated plasmid delivery in animals. *Technol. Cancer Res. Treat.* 1: 365–371.
50. Rodenburg, J. (1996). Body condition scoring of cattle. Ontario Ministry of Agriculture and Food Fact Sheet, pp. 92–122.
51. Davis, W. C., Davis, J. E., and Hamilton, M. J. (1995). Use of monoclonal antibodies and flow cytometry to cluster and analyze leukocyte differentiation molecules. *Methods Mol. Biol.* 45: 149–167.
52. Larsen, R. A., Monaghan, M. L., Park, Y. H., Hamilton, M. J., Ellis, J. A., and Davis, W. C. (1990). Identification and characterization of monoclonal antibodies reactive with bovine, caprine and ovine T-lymphocyte determinants by flow microfluorimetry. *Vet. Immunol. Immunopathol.* 25: 195–208.
53. Davis, W. C., MacHugh, N. D., Park, Y. H., Hamilton, M. J., and Wyatt, C. R. (1993). Identification of a monoclonal antibody reactive with the bovine orthologue of CD3 (BoCD3). *Vet. Immunol. Immunopathol.* 39: 85–91.
54. Baldwin, C. L., Teale, A. J., Naessens, J. G., Goddeeris, B. M., MacHugh, N. D., and Morrison, W. I. (1986). Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonal antibodies and function: similarity to lymphocytes defined by human T4 and murine L3T4. *J. Immunol.* 136: 4385–4391.
55. Davis, W. C., Hamilton, M. J., Park, Y. H., Larsen, R. A., Wyatt, C. R., and Okada, K. (1990). Ruminant leukocyte differentiation molecules. In: *Monographs in Animal Immunology: MHC, Differentiation Antigens and Cytokines in Animals and Birds* (O. Barta, Ed.), pp. 47–70. Bar-Lab, Blacksburg, VA.
56. Naessens, J., Sileghem, M., MacHugh, N., Park, Y. H., Davis, W. C., and Toye, P. (1992). Selection of BoCD25 monoclonal antibodies by screening mouse L cells transfected with the bovine p55-interleukin-2 (IL-2) receptor gene. *Immunology* 76: 305–309.
57. Lee, S. U., *et al.* (2001). Identity of activation molecule 3 on superantigen-stimulated bovine cells is CD26. *Infect. Immun.* 69: 7190–7193.
58. Bembridge, G. P., Howard, C. J., Parsons, K. R., and Sopp, P. (1993). Identification of monoclonal antibodies specific for bovine leukocyte common antigen (CD45) together with a novel broadly expressed leukocyte differentiation antigen, BoWC11. *Vet. Immunol. Immunopathol.* 39: 115–120.
59. Davis, W. C., *et al.* (2001). Analysis of monoclonal antibodies reacting with molecules expressed on gammadelta T-cells. *Vet. Immunol. Immunopathol.* 80: 53–62.
60. Hein, W. R., Dudler, L., and Mackay, C. R. (1989). Surface expression of differentiation antigens on lymphocytes in the ileal and jejunal Peyer's patches of lambs. *Immunology* 68: 365–370.
61. Alvarez, B., *et al.* (2000). A porcine cell surface receptor identified by monoclonal antibodies to SWC3 is a member of the signal regulatory protein family and associates with protein-tyrosine phosphatase SHP-1. *Tissue Antigens* 55: 342–351.